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ZFAT expression in B and T lymphocytes and identification of ZFAT-regulated genes

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Abstract

The human *ZFAT* gene encodes a 1243-amino-acid protein containing one AT hook and 18 C2H2 zinc finger domains, which are highly conserved among ZFAT orthologues from fish to mammalian species. Consistent with the presence of multiple predicted nuclear localization signals, endogenous ZFAT protein was found to be localized to the nucleus. In the mouse tissues examined by Western blotting, ZFAT was found to be expressed in thymus, spleen, and lymph nodes, but not in other tissues, including bone marrow. Furthermore, ZFAT protein was found to be up-regulated during the transition from $CD4^-CD8^-$ to $CD4^+CD8^+$ thymocytes and to be expressed only in B and T lymphocytes in peripheral lymphoid tissues. Expression array analyses demonstrated that genes that are down-regulated upon ZFAT overexpression in mouse Ba/F3 cells are significantly enriched for those functionally related to immune responses. These results suggest that ZFAT functions as a critical transcriptional regulator in B and T lymphocytes.

Keywords: ZFAT; C2H2 zinc finger; AT-hook; Lymphocytes; Microarray; Gene Ontology; Autoimmune thyroid diseases

Proteins with zinc finger domains constitute one of the largest classes of protein superfamily in the mammalian genome and are best characterized as transcriptional regulators in a variety of cellular activities [1]. The C2H2 zinc finger is the most common form among over 80 subclasses of zinc finger domains (the list of Pfam entries is available at http://pfam.sanger.ac.uk/). One zinc finger, composed of 25-30 amino acid residues, recognizes the major groove of the double-helix DNA and interacts with 3 bp. Tandem repeats of multiple zinc fingers determine the sequence specificity of DNA recognition [1]. In the SMART database (http://smart.embl-heidelberg.de/), 789 human and 760 mouse proteins with C2H2 zinc finger domains are registered. While many of these zinc finger proteins have already been well characterized, a large portion of them are still functionally unknown. Assignment of functional information to each of uncharacterized zinc finger proteins certainly facilitates the understanding of their roles in various cellular processes and potential relevance to human diseases.

Autoimmune thyroid disease (AITD), including Graves' disease (GD) and Hashimoto thyroiditis (HT), is caused by an immune response to self-thyroid antigens and affects up to 2-5% of the general population [2]. GD is characterized by the production of thyroid-stimulating hormone receptor-stimulating antibodies, leading to hyperthyroidism, whereas HT is characterized by the apoptosis of the thyrocytes, resulting in hypothyroidism [3,4]. Twin studies and familial aggregation have clearly indicated that AITD is caused by a combination of both environmental and genetic factors [5,6]. Through linkage and association analyses on a cohort of Japanese AITD patients [7,8], we have previously identified *ZFAT* (zinc-finger gene in

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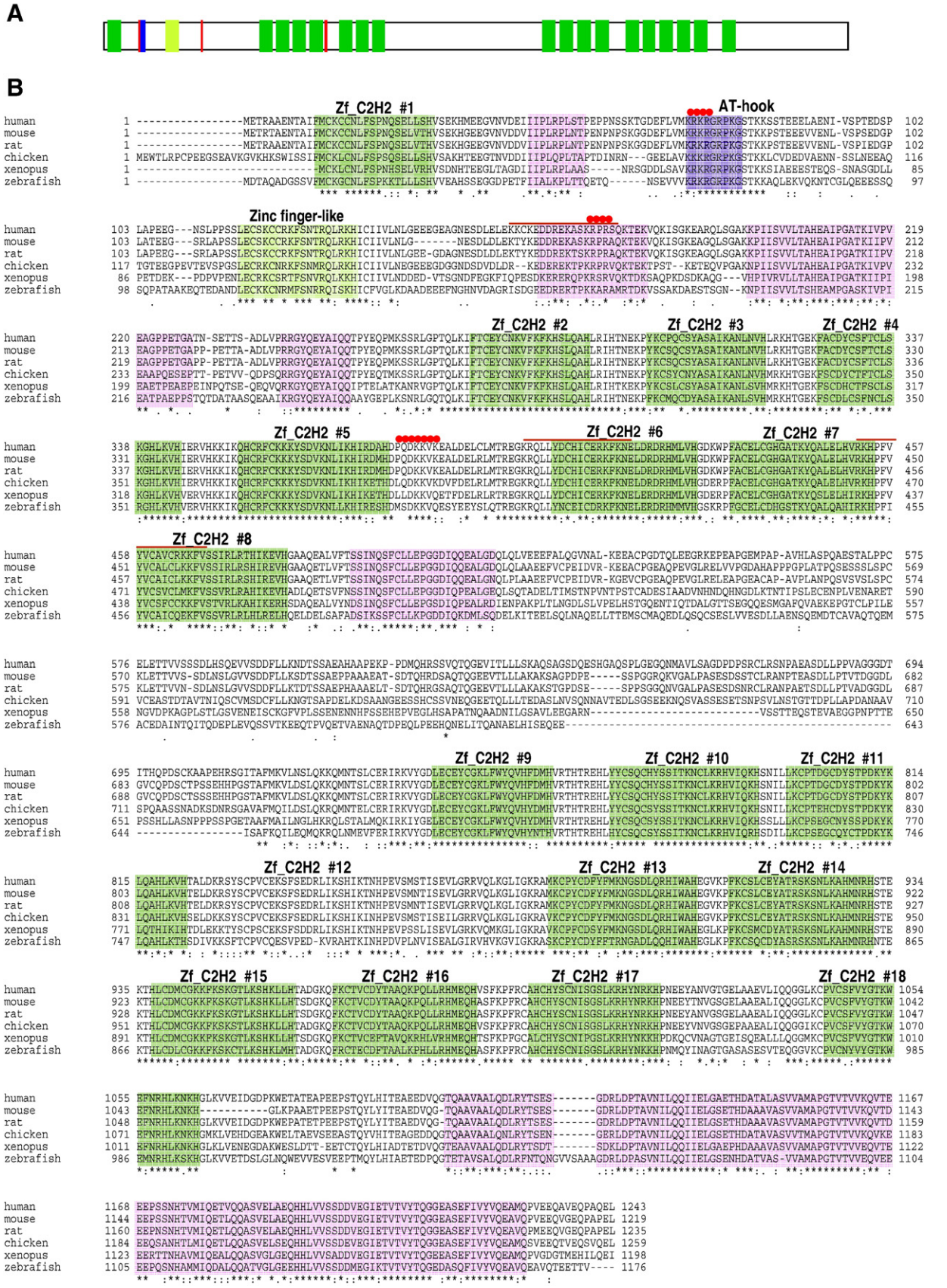
A
B
Zf_C2H2 #1
AT-hook
Zinc finger-like
Zf_C2H2 #2
Zf_C2H2 #3
Zf_C2H2 #4
Zf_C2H2 #5
Zf_C2H2 #6
Zf_C2H2 #7
Zf_C2H2 #8
Zf_C2H2 #9
Zf_C2H2 #10
Zf_C2H2 #11
Zf_C2H2 #12
Zf_C2H2 #13
Zf_C2H2 #14
Zf_C2H2 #15
Zf_C2H2 #16
Zf_C2H2 #17
Zf_C2H2 #18
human
mouse
rat
chicken
xenopus
zebrafish

AITD susceptibility region) on 8q24 as a susceptibility gene for both GD and HT [8].

The human *ZFAT* gene, also known as *ZNF406,* encodes a 1243-amino-acid residue protein that contains one AT hook and 18 C2H2 zinc-finger domains. The AT-hook domain is composed of a conserved 9-amino-acid DNA-binding motif containing a GRP consensus sequence [9]. It was originally identified in the HMG-I(Y) protein and has been found in a wide variety of DNA binding proteins. It has been shown to be necessary and sufficient to bind to the minor groove of AT-tract DNA [10,11]. These domain features indicate the possibility that ZFAT protein binds to DNA and functions as a transcriptional regulator. Here, we show that ZFAT protein is expressed in B and T lymphocytes in mouse and that ZFAT regulates immune-related genes functionally important for immune responsiveness.

Results and discussion

Evolutionary conservation of domain contents of ZFAT

The human ZFAT protein contains an AT-hook domain at the N-terminal region and 18 C2H2-type zinc finger domains. Eight zinc finger domains (and a zinc finger-like domain) are located in the N-terminal-half region and the remaining 10 zinc finger domains in the C-terminal-half region (Fig. 1A). To observe the evolutionary conservation of ZFAT protein structure in multiple lineages, we collected nucleotide and protein sequences from a wide range of organisms using genome annotation databases and the BLAST programs (NCBI). Based on the similarity of the deduced protein sequences and the conservation of exon-intron structures of the collected gene sequences, we concluded that mammalian species, chicken, lizard, frog, and at least some of teleost fish species contain a gene orthologous to the human *ZFAT* gene. No apparent paralogous gene with *ZFAT* was found in these vertebrate species. A multiple alignment of protein sequences from human, mouse, rat, chicken, *Xenopus,* and zebrafish was generated for sequence comparisons (Fig. 1B). The overall content, the positions, and the sequences of the zinc finger and AT-hook domains were well conserved among the six species. The perfect conservation between human and zebrafish was observed for the second zinc finger and the AT-hook domains. As for the regions outside of the known domains, several evolutionarily highly conserved regions (highlighted in pink in Fig. 1B) and one continuous highly diverse region between zinc fingers 8 and 9 were observed.

PSORTII programs (http://psort.nibb.ac.jp/form2.html) predicted three classical and three bipartite nuclear localization signals (NLSs) in the human ZFAT protein sequence. The predicted classical NLSs are ^{71}KRKR^{74} overlapped with the AT-hook motif (KRKRGRPKG), ^{171}KRPR^{174}, and ^{379}PQDKKVK^{385}. The predicted bipartite NLSs are ^{159}KKCKEDDREKASKRPRS^{175}, ^{399}KRQLLYDCHICERKFKN^{415}, and ^{452}RKHPFVYVCAVCRK KFV^{468}. All six NLSs are mostly evolutionarily conserved and positioned in the N-terminal-half region. Consistent with this prediction, when the ZFAT protein with a HA tag at its C-terminus was transiently expressed in HEK293 cells, the protein was found to be predominantly localized to the nucleus (data not shown).

These observations indicate that ZFAT likely binds to DNA through its evolutionarily conserved AT-hook and at least some of the C2H2 zinc-finger domains and functions as a transcriptional regulator in the nucleus. The conserved regions outside the known domains may be involved with transactivation/repression and/or protein-protein interactions and should be further characterized in future studies. The evolutionarily diverse region may function as a spacer to separate two zinc finger clusters, which are potentially functionally distinct from each other as was shown in previously well-characterized multi-zinc finger-containing proteins such as the Ikaros family [14] and CTCF [15]. We are currently performing chromatin immunoprecipitation analysis to identify direct ZFAT target sites in the mouse genome.

Expression pattern of the endogenous ZFAT protein in mouse tissues and purified lymphocytes

We raised rabbit polyclonal antiserum against a 164-residue C-terminal fragment of the mouse ZFAT protein. The polyserum detected a major band at approximately 180 kDa on mouse Ba/F3 and other cell lines in Western blot analysis (data not shown). The intensity of the detected band was significantly decreased on the Ba/F3 cells treated with small inhibitory RNAs (siRNAs) targeted to *Zfat,* compared to the Ba/F3 cells treated with scramble control RNAs, indicating that the detected band by the polyserum is specific to ZFAT protein (Fig. 2A). We then determined the tissue and cell-type distribution of ZFAT protein. In the nine tissues from 6-week-old male C57BL6/J mice tested, the ZFAT protein was detected in spleen and thymus, but not in other tissues, including bone marrow (Fig. 2B). Subsequently, we examined MACS-selected lymphocytes from immune tissues of C57BL6/J mice (6-week-old, male) to determine the cell-type distribution of the ZFAT protein. Among lymphocytes

Fig. 1. (A) Domain/motif content of the human ZFAT protein. The positions of an AT-hook motif, a zinc finger-like domain, C2H2 zinc finger domains (Zf_C2H2 #1-18), and three predicted classical NLSs [16] are shown as blue, light green, green, and red rectangles, respectively. (B) The multiple alignment of ZFAT orthologues from six species. Six protein sequences (NP_065914.2 (human), NP_941046.1 (mouse), XP_343256.2 (rat), XP_418429.2 (chick), NP_001073041.1 (*Xenopus*), and XP_694698.2 (zebrafish)) were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html). The degree of conservation is denoted by the following symbols: "*" (identical in all sequences), ":" (conserved substitutions), and "." (semiconserved substitutions). Each of the C2H2 zinc finger domains (highlighted in green) were defined by the pattern of #-X-C-X(1-5)-C-X3-#-X5-#-X2-H-X(3-6)-[H/C], where X can be any amino acid, numbers in parentheses indicate the number of residues, and # are those that are important for the stable fold (Pfam Accession No. PF00096, http://www.sanger.ac.uk/Software/Pfam/). Highly conserved regions outside the clusters of zinc finger domains (#2-8 and #9-18) are highlighted in pink. The positions of predicted classical and bipartite NLSs are shown by red dots and bars, respectively.

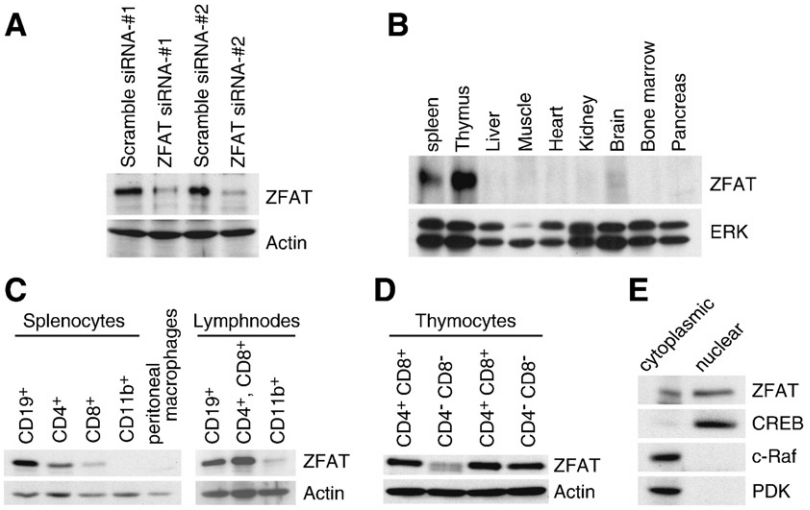

Fig. 2. Western blot analyses for (A) siRNA-treated and control mouse Ba/F3 cells, (B) nine types of tissues from 6-week-old male C57BL/6J mice, (C) mouse peritoneal macrophages and MACS-selected lymphocytes isolated from mouse spleen and lymph nodes, (D) MACS-selected lymphocytes isolated from mouse thymus, and (E) cytoplasmic and nuclear fractions of Ba/F3 cells. Affinity-purified anti-ZFAT antibody was used to detect endogenous ZFAT protein. Western blotting using anti-actin antibody or anti-ERK antibody was performed as loading control (A-E).

isolated from spleen and lymph nodes, ZFAT was detected in $CD19^+$, $CD4^+$, and $CD8^+$ lymphocytes, but undetectable in $CD11b^+$ lymphocytes (Fig. 2C). ZFAT protein was also undetectable in mouse peritoneal macrophages. These results demonstrated that ZFAT protein is predominantly expressed in B and T lymphocytes but not in other cell types such as monocytes and macrophages in secondary lymphoid tissues. To assess the expression pattern of ZFAT protein during thymocyte differentiation, we separated thymocytes into four fractions ($CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$) and subjected them to Western blot analysis. ZFAT was found to be expressed in immature $CD4^-CD8^-$ double-negative cells at a low level and expressed at much higher levels in the other three fractions (Fig. 2D). To determine the subcellular localization of endogenous ZFAT protein, cytoplasmic and nuclear fractions from Ba/F3 cells were analyzed. Consistent with the aforementioned result of the transient expression assay, the endogenous ZFAT protein was detected in the nuclear fraction, although a significant amount of ZFAT protein was also detected in the cytosolic fraction (Fig. 2E). The nuclear-specific detection of CREB and the cytoplasmic-specific detection of c-Raf and PDK indicate that the subcellular fractionation was performed successfully.

Taken together with the fact that ZFAT protein was undetectable in bone marrow, where immature B lymphocytes and thymus-seeding progenitor cells reside, the expression level of ZFAT protein seems to be kept low at early stages and to become elevated at later stages during the differentiation of the B and T cell lineages. At the mRNA level, ZFAT expression could be detected in a wide variety of tissues at various levels [8]. Therefore, it was intriguing that the expression of mouse ZFAT protein was found to be limited to spleen, lymph nodes, and thymus. This observation indicates the possibility that a posttranslational regulation mechanism(s) to stabilize or to degrade the ZFAT protein exist in these immune tissues. Our results imply that ZFAT functions as a transcriptional regulator during B and T lymphocyte differentiation.

Global gene expression analysis using expression microarrays

As an approach to understanding the immunological functions of ZFAT, we examined the impact of ZFAT overexpression on the global gene expression in a mouse immune cell line, Ba/F3. Three Ba/F3 clones that stably express ZFAT-HA protein, as well as three control clones carrying an empty vector, were isolated and subjected to microarray-based expression analyses. The ectopic expression of ZFAT-HA was confirmed by quantitative RT-PCR (data not shown) and Western blot analysis (Fig. 3A). We initially employed CodeLink UniSet Mouse 20K bioarrays to identify genes that were differentially expressed upon ZFAT overexpression. We used two filtering methods, volcano plots and Venn diagrams, with two different thresholds for each method to select differentially expressed genes. The number of the genes selected from the 12,982 "present/good" genes varied from 805 (6.2%) to 50 (0.39%) among four types of the selections performed (Table 1). The list of the 216 genes (1.66%) selected by volcano plot filtering (fold change >2.0 and t test $p<0.1$), composed of 171 down-regulated and 45 up-regulated genes, is provided as Supplementary Tables 1 and 2. Among the 50 differentially expressed genes selected using Venn diagrams with 2.0-fold change as the threshold, 24 genes were subjected to quantitative RT-PCR to validate the CodeLink data. Up- or down-regulation identified by the CodeLink arrays was reproduced for the majority of the genes examined (Fig. 3B and Supplementary Tables 1 and 2).

Furthermore, we analyzed the same materials using the GeneChip Mouse Genome 430 2.0 array (Affymetrix) and extracted differentially expressed genes from the 20,218 "present/marginal" genes (Table 1). The number of differentially expressed genes varied from 219 (1.09%) to 24 (0.12%). The

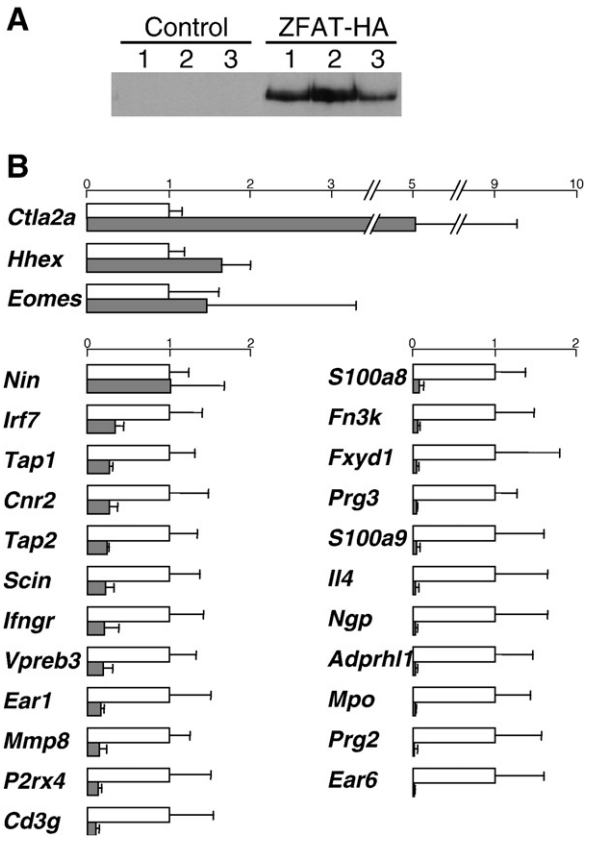

Fig. 3. (A) Western blot analysis using an anti-HA antibody (3F10) on ZFAT-HA stable transformants and control Ba/F3 clones. (B) The expression levels of 24 differentially regulated genes in ZFAT-HA stable transformants (gray bars) relative to those in control Ba/F3 clones (open bars) measured by quantitative RT-PCR. Three genes (*Ctla2, Hhex,* and *Eomes*) were identified as up-regulated, and the other genes as down-regulated, by the CodeLink arrays.

list of the 219 genes selected by volcano plot filtering (fold change >1.5 and *t* test $p<0.1$), composed of 120 down-regulated and 99 up-regulated genes, is provided as Supplementary Tables 3 and 4. Comparison of the number of differentially expressed genes between CodeLink and Affymetrix datasets with the same filtering condition revealed that 3.2-6.3 times higher percentage of the genes were detected in the CodeLink dataset than in the Affymetrix dataset. This tendency may have originated from the lower noise levels of the CodeLink array compared to the Affymetrix GeneChip at middle to low expression ranges [18].

To search for common functional features among these ZFAT-regulated genes, we performed Gene Ontology (GO) analysis using the GeneSpring software. To compare the results between CodeLink and Affymetrix datasets, the 216 differentially expressed genes from the CodeLink data and the 219 genes from the Affymetrix data (Table 1) were subjected to GO analysis, since these two sets are almost the same in the number of genes detected. Interestingly, GO terms showing statistically significant enrichment ($p<10^{-5}$) were identified only in the down-regulated genes in both datasets (Tables 2 and 3). All GO terms identified are related to keywords such as "immune/inflammatory response," "response to external stimulus," or "chemotaxis." Such GO terms were also found to be associated with the sets of down-regulated genes selected by other criteria (Table 1) (data not shown). Although only 21 genes (underlined in Supplementary Tables 1-4) are common between 216 and 219 differentially expressed genes identified by two employed platforms, GO analysis captured extremely similar functional signatures between the two datasets. This is consistent with the previous observation that enriched GO terms showed the highest reproducibility in the systematic comparisons of the datasets acquired using different microarray platforms for standard RNA samples [17]. The down-regulated genes to which the enriched GO terms listed in Tables 2 and 3 are assigned (shown in boldface in Supplementary Tables 1-4) include cell surface receptor genes (such as *Fcgr2b, Ccr1, Ccr2, C3ar1, Fcrg2b,* and *Olr1*), ligand genes (such as *Il4, Il15, Il1rn, Mst1, Ccl6, Ccl9,* and *Cxcl15*), and secreted protein-coding genes (such as *Prg2, Ngp, S100b, S100a8, S100a9, Pglyrp1, Cfp,* and *Spp1*). The encoded proteins by these genes are all associated with cell membrane and/or extracellular matrix, and many of those are involved in immune cell signaling. Therefore, down-regulation of these genes by ZFAT overexpression may make Ba/F3 cells inert against external immune stimuli.

In summary, we have revealed that ZFAT protein is expressed exclusively in B and T lymphocytes in immune tissues in differentiation stage-specific manners and that overexpression of ZFAT in Ba/F3 cells mainly down-regulates a subset of genes functionally important in immune response. Since ZFAT was originally identified as an AITD susceptible gene, our findings

Table 1
Number of genes differentially expressed upon ZFAT overexpression identified using two array platforms

Platform	Expression	No. of genes (%)			
		Volcano plot		Venn diagram	
		Fold change >1.5 ($p<0.1$)	Fold change >2.0 ($p<0.1$)	Fold change >1.5	Fold change >2.0
CodeLink UniSet Mouse 20K bioarray (12,982 genes)	Down	467 (3.60%)	171 (1.32%)	152 (1.17%)	46 (0.35%)
	Up	338 (2.60%)	45 (0.35%)	44 (0.34%)	4 (0.03%)
	Total	805 (6.20%)	216 (1.66%)	196 (1.51%)	50 (0.39%)
Affymetrix GeneChip Mouse Genome 430 2.0 (20,018 genes)	Down	120 (0.60%)	42 (0.21%)	41 (0.20%)	22 (0.11%)
	Up	99 (0.49%)	21 (0.10%)	8 (0.04%)	2 (0.01%)
	Total	219 (1.09%)	63 (0.31%)	49 (0.24%)	24 (0.12%)

Table 2
Gene Ontology terms enriched in ZFAT-regulated genes identified using CodeLink arrays

Category	Genes in category (10,941)		Down-regulated (125 genes)			Up-regulated (34 genes)		
	n	%	*n*	%	*p* value	*n*	%	*p* value
GO:6955, immune response	560	5.1	21	16.8	$\mathbf{1.29\times10^{-6}}$	3	8.8	0.25
GO:6952, defense response	639	5.8	22	17.6	$\mathbf{2.88\times10^{-6}}$	2	5.9	0.60
GO:9607, response to biotic stimulus	670	6.1	22	17.6	$\mathbf{6.20\times10^{-6}}$	2	5.9	0.63

Of 171down-regulated and 45 up-regulated genes, 125 and 34, respectively, were found to have GO term annotations and were subjected to the GO term enrichment analysis.

facilitate the understanding of ZFAT functions in immunity and related diseases.

Materials and methods

Cloning of full-length mouse Zfat cDNA

A 1.6-kb mouse *Zfat* cDNA fragment was amplified by a PCR primer set for human *ZFAT* (5'-ACCAGTTCCATCAACCAGAGC-3' and 5'-AAAGCTGCAAACAGGACACTT-3') using mouse small intestine cDNA as template. 5'- and 3'-RACE was performed using the SMART RACE cDNA amplification kit (Clontech) to obtain full-length cDNA. The sequence of the amplified cDNA fragments by RACE contained the first Met and the termination codon. The complete open reading frame sequence of *Zfat* (GenBank Accession No. EU221277) was amplified by PCR and cloned.

Cell culture and siRNA

Ba/F3 cells were cultured at 37°C with 5% CO_2 in RPMI 1640 containing 10% fetal calf serum and 10% WEHI3B conditioned supernatant. Ba/F3 cells were transfected with a siRNA by electroporation. Two distinct siRNAs were designed to target the mouse *Zfat* mRNA sequence (nt 231-255 and 740-764 of GenBank Accession No. NM_198664). Scrambled RNAs containing the same number of each nucleotide as the siRNAs targeted to *Zfat* were used as controls.

The following siRNA duplexes were used: Zfat #1, 5'-AAACGUGGGUCACGAGUUCUGACUG-3' and 5'-CAGUCAGAACUCGUGACCCACGUUU-3'; scramble RNA #1, 5'-AAAGACGUGGGUCACGAGUUCUCUG-3' and 5'-CAGAGAACUCGUGACCCACGUCUUU-3'; Zfat #2, 5'-UUCAUGAGCAGUUAAGACCACGCUG-3' and 5'-CAGCGUGGUCUUAACUGCUCAUGAA-3'; scramble RNA #2, 5'-UUCGCAGAGUUGACGAAUACCACUG-3' and 5'-CAGUGGUAUUCGUCAACUCUGCGAA-3'.

Isolation of ZFAT-HA stable transformants

A 3.7-kb fragment containing the full-length mouse *Zfat* coding region (nt 1-3711 of GenBank Accession No. EU221277), a HA-tag sequence at the C-terminus of ZFAT, and a stop codon right after the HA-tag sequence was cloned into an expression vector carrying the CMV enhancer-promoter. Ba/F3 cells (5×10^6) were transfected with 15 μg of the plasmid DNA by electroporation. The pulsed cells were disseminated into 96-well plates and incubated in the selection medium supplemented with 1 mg/ml G418. The expression level of mouse ZFAT-HA was tested by Western blot using anti-HA antibody (3F10; Roche). Stable transformants expressing ZFAT-HA were subcloned by limiting dilution.

Generation of anti-ZFAT polyserum and Western blot analysis

Recombinant mouse ZFAT fragment (amino acid residues 1074-1237) was expressed as a GST fusion protein using the pGEX6P-1 vector (GE Healthcare). The fusion protein was soluble in nondenaturing buffer and was purified with glutathione-Sepharose4B. Antiserum was obtained by injecting the recombinant ZFAT protein into a Japanese White rabbit followed by booster injection. Antiserum was purified with an affinity column prepared by cross-linking the recombinant protein to CNBr-activated Sepharose 4B. Positive selection or depletion of a specific type of lymphocyte was carried out using MACS microbeads coated with a specific monoclonal antibody (Miltenyi Biotec). Tissues from C57BL/6J mice, purified lymphocytes, or cultured cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche)) and subjected to Western blotting as described previously [12]. Antibodies used were affinity-purified anti-ZFAT antibody (dilution 1:1000), anti-ERK1 antibody (K-23; Santa Cruz Biotechnology; 1:1000), anti-CREB antibody (Cell Signaling; 1:500), and anti-actin antibody (H-300; Santa Cruz Biotechnology; 1:1000).

Expression array analyses and quantitative RT-PCR

Total RNA was extracted from three ZFAT-HA stable transformants and three control Ba/F3 clones using the Qiagen RNeasy kit. Preparation of biotinylated antisense cRNA, hybridization to the CodeLink UniSet Mouse 20K bioarray (GE Healthcare) and to the GeneChip Mouse Genome 430 2.0 array (Affymetrix), washing, scanning, and data processing were performed according to the manufacturers' instructions and as described previously [13]. Output files were then loaded into GeneSpring v7.3 (Agilent Technologies) with per-chip

Table 3
Gene Ontology terms enriched in ZFAT-regulated genes identified using GeneChip arrays

Category	Genes in category (20,834)		Down-regulated (56 genes)			Up-regulated (43 genes)		
	n	%	*n*	%	*p* value	*n*	%	*p* value
GO:9605, response to external stimulus	792	3.8	16	28.6	$\mathbf{1.76\times10^{-10}}$	1	2.3	0.81
GO:9613, response to pest, pathogen, or parasite	525	2.5	13	23.2	$\mathbf{1.06\times10^{-9}}$	1	2.3	0.67
GO:43207, response to external biotic stimulus	538	2.6	13	23.2	$\mathbf{1.43\times10^{-9}}$	1	2.3	0.68
GO:6954, inflammatory response	212	1.0	9	16.1	$\mathbf{5.15\times10^{-9}}$	0	0.0	1.00
GO:6955, immune response	854	4.1	14	25.0	$\mathbf{4.21\times10^{-8}}$	2	4.7	0.53
GO:6952, defense response	1022	4.9	15	26.8	$\mathbf{5.40\times10^{-8}}$	3	7.0	0.36
GO:9607, response to biotic stimulus	1052	5.1	15	26.8	$\mathbf{7.89\times10^{-8}}$	3	7.0	0.37
GO:42330, taxis	157	0.8	7	12.5	$\mathbf{2.12\times10^{-7}}$	0	0.0	1.00
GO:6935, chemotaxis	157	0.8	7	12.5	$\mathbf{2.12\times10^{-7}}$	0	0.0	1.00
GO:9611, response to wounding	382	1.8	9	16.1	$\mathbf{7.86\times10^{-7}}$	0	0.0	1.00
GO:42221, response to chemical stimulus	435	2.1	9	16.1	$\mathbf{2.29\times10^{-6}}$	1	2.3	0.60

Of 120 down-regulated and 99 up-regulated genes, 56 and 43, respectively, were found to have GO term annotations and were subjected to the GO term enrichment analysis.

normalization to the 50th percentile and per-gene normalization to the average expression level of the control BaF3 clones. Genes that were differentially expressed upon ZFAT overexpression were selected using two independent filtering methods. One method was volcano plot filtering, in which the relative fold change between the mean values of triplicates (three ZFAT-HA transformants and three controls) and the statistical significance level (p value for a t test of difference between the two groups) are considered. The other was the use of Venn diagrams to select genes that consistently exceed a designated threshold (2.0- or 1.5-fold change) in all of three ZFAT-HA transformants compared to each of three controls.

Quantitative RT-PCR was performed using LightCycler and FastStart DNA Master SYBR Green I (Roche). The thermal cycling conditions were initial denaturation at 95°C 5 min, followed by up to 40 cycles of denaturation at 95°C for 15 s, annealing at 62°C for 15 s, and extension at 72°C for 10 s. The PCR primer sequences used for each of 24 genes are listed in Supplementary Table 5. Absolute quantification was performed for each gene and for the β-actin gene as the normalization standard. The fold change of the mean of three ZFAT-HA transformants relative to that of three controls was determined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2008.01.009.

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